IN VITRO ANTIBACTERIAL ACTIVITY OF *Vernonia amygdalina* LEAVES EXTRACTS AGAINST PATHOGEN OF PINEAPPLE HEART ROT DISEASE

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Abstract

Heart rot disease is one of the major diseases in pineapple cultivation in Malaysia. Josapine and N36 are among varieties that vulnerable to the heart rot disease. The infection can reduce up to 50% yield. *In vitro* antibacterial activity of four *Vernonia amygdalina* leaves extract (hexane, chloroform, methanol and aqueous) was carried out and evaluated against pathogen of pineapple heart rot disease. The antibacterial activity was evaluated using well diffusion method where three concentrations for each extract were prepared. The results revealed *V. amygdalina* chloroform and aqueous leaves extracts at 10000 ppm shows greater inhibition zone than positive control. *V. amygdalina* hexane leaves extracts, however, did not shows any inhibition zone at any concentrations. Meanwhile, the *V. amygdalina* methanol leaves extract shows antibacterial activity against the pathogen of pineapple heart rot disease but not as good as positive control. Phytochemical screening was also carried out to determine the presence of alkaloid, flavanoid, glycoside, phenol, saponin, steroid, and tannins as these compounds may attribute to the result. The screening revealed that both *V. amygdalina* chloroform and aqueous leaves extracts contain alkaloid, saponin, and tannin. Result shows the potentials of *V. amygdalina* as an antibacterial agent in combating pineapple heart rot disease.

Keywords: antibacterial activity, inhibition zone, phytochemical screening, pineapple heart rot disease, *Vernonia amygdalina*

Introduction

In Malaysia, pineapple (*Ananas comosus*) is grown for its fresh and canned fruit. Placed at sixth place for the worldwide canned pineapple exporting country, Malaysia export about 44000 tonne canned pineapple per year (Rohrbach et al., 2003). To meet the demand, the pineapple plantation area is increased every year, from 5400 ha in 2001 to 15100 ha in 2005. Nowadays, average about 13000 ha land was planted with pineapple especially in Johor, Malaysia. Mauritius (Moris), Sarawak, Josapine and Maspine are among commercial pineapple variety planted in Malaysia. Pineapple not only popular as can fruit but also

marketed as fresh fruits. Every year, pineapple fresh fruit give significant earning for Malaysia agriculture production (Ramachandran et al., 2015).

The pineapple productivity, however, has been decline as its vulnerable and susceptible to the attacks by pathogen. By far, heart rot disease is one of the bacterial disease that attack and cause serious damage and losses in pineapple productivity. Plant pathogenic bacteria are responsible for major economic losses in agricultural worldwide (Stavrinides et al., 2011). Heart rot disease infestation happen during fruit development with low light conditions (Abdullah & Cheng, 2011). The infestation symptoms can be seen through water-soaked lesions on the locus of leaves. Brown streaks were formed in the lamina and mesophyll tissue. Light brown streaks can be seen emerges from the blisters when the leaves start to rot. The pathogen of heart rot disease is aggressively moves from leaves to the heart of the pineapple and give stress to the plants. After a few days of infections, the pineapple heart and stem can be easily to fall down.

The heart disease is known to be caused by *Erwinia chrysanthemi.* The *Erwinia chrysanthemi* is recently known as *Dickeya zeae* (Peckham et al., 2010; Marrero & Alvarez, 2011). Virulence is related to the ability of *E. chrysanthemi* strains to produce pectinase that are able to break down the plant cell. It is commonly infected at plants include potato tubers, bulbs of vegetables and ornamental crops (Perombelon & Kelman, 1980). Pineapple heart rot disease first occurs in Hawaii in 2003 and reoccured in Malaysia in 2006 (Kaneshiro et al., 2008). The incident of pineapple heart rot disease was reported in Tanah Merah, Pasir Mas, Kuala Ketil and Perak which caused the declining of yield up to 50%. The most susceptible pineapple varieties to heart rot disease were the variety of Josapine and N36 which could cause losses up to 64% (Sahilah et al., 2008).

The most common and effective control method for pineapple heart rot disease that being used was by using chemical pesticides. Recently, more than 800 active ingredients in a wide range of commercial pesticide are registered for the use in agriculture to meet source of food for our nutrition (Stoytcheya, 2011). Malathion is one of the synthetic pesticides used to control heart rot disease. Even though the application of synthetic pesticides give fast result and can control the disease outbreak, the application still has the adverse effect such as the development of pathogenic bacteria resistance, harmful to the environment, wildlife and human. The life-long health problems, birth defects, and cancers are among the impact of pesticide usage to human being (Bhuvaneswari & Valasundaram, 2009).

The awareness of the harmful effect has changed the consumer's preferences and demand on quality food and reducing the usage of synthetic pesticides. The concern on healthy diets and environmental issues has direct the researcher to produce safer and eco-friendly pesticides for controlling the plant disease. As plant itself has their own defense mechanism from pathogen infection, plant extract is one of good choice to be developed as biopesticides. The plant extract from seeds, leaves, and bark of *Amorphophallous companulatus, Azadirachta indica, Gnidia glauca, Pongamia pinnata and Strychnus nuxvomica* has been proved can be used to control pineapple heart rot disease (Naik & Maheswarappa, 2007).

In this study, *V. amygdalina,* a member of Asteracea family were chosen to evaluate its *in vitro* antibacterial activity against pathogen of pineapple heart rot disease. *V. amygdalina* grows well in tropical Africa and can grow up to 3 meters with petiolate leaf (Ebenezer & Olatunde, 2011). *Pokok Bismillah* and *Pokok Afrika Selatan* are among the common name of

V. amydalina. *V. amygdalina* has been proved to have terpenes, steroids, coumarins and lignans. It is known for its bitter taste, due to anti-nutritional factors compounds such as alkaloids, saponin and tannin. *V. amygdalina* is a multipurpose plant that has a lot of potentially value in medicinal properties. It has potential as antioxidants and food preservatives (Swee et al., 2010; Loliger, 1991), antibacterial activity (Ijeh et al., 1996), and antimalarial activity (Madureira et al., 2002).

Materials and Methods

Plant Extraction

Plant material *(V. Amygdalina* leaves) was collected, air-dried, ground and stored in airtight container. The plant material was then extracted via sequential extraction where it has been macerated with hexane, chloroform and methanol for 24 hours. The maceration then filtered and evaporated using rotary evaporator. Three different concentrations were prepared (1000 ppm, 5000 ppm, 10000 ppm) for each extract.

Method for aqueous extraction was based on the method applied previously (Thamer, 2008). The dried sample was added to distilled water and mixed with electrical mixer and then filtered. The filtrate was then separated by centrifuge. The precipitate was put in petri dish and kept in oven at 45^{o}C. The extract was kept for future use. Three different concentrations were also prepared for aqueous extract.

Isolation of Pineapple Heart Rot Pathogen

The bacteria strain was isolated from heart rot disease of pineapple by followed serial dilution method. About 1 mL of suspension from each dilution was streaked onto nutrient agars. The inoculated plates were kept in incubator at 37^{o}C for 24 hours and observed for colony growth. Single colonies were collected and transferred onto sterile nutrient agar. The sub-culture plates were examined and maintain for its viability. Morphology and microscopic observation were done to identify the colony appearance, morphology and gram staining properties.

In Vitro Antibacterial Activity Test

Agar and broth media were prepared for the *in vitro* antibacterial activity test. Both agar and broth dilution were sterile using autoclave at 151^{o}C for 15 minutes. The agar media were then poured into petri dish and left to cool. Both media were kept for future used.

The *in vitro* antibacterial activity test was evaluated using well diffusion methods (Magaldi et al., 2004; Valgas et al., 2007). The isolated bacteria were spread evenly in agar media. Two 5 mm wells were made and 50µl of plant extract were pipetted into wells. The positive control, negative control and extracts were placed on inoculated plate. Standard antibiotic, gentamicin was used as a positive control while well containing $DMSO_4$: methanol (for hexane and chloroform extracts) and 50% methanol (for methanol and aqueous extracts) were used as negative control. Plates were incubated for 24 hours at 37^{o}C. The clear zones indicate the inhibition zone of bacterial growth were measured.

Phytochemical Screening Test

The qualitative screening was carried out for determining the presence of alkaloids, flavonoids, glycosides, phenol, saponin, steroids and tannin as suggested previously (Yusuf et al., 2014; Rios & Recio, 2005; Egwaikhide & Gimba, 2007; Edeoga et al., 2005).

Alkaloids: The presence of alkaloids in *V. amygdalina* was detected using Dragendorff's test. 1ml of Dragendorff's reagent was added drop by drop onto the plant extracts. Formation of reddish-brown precipitate indicates the presence of alkaloids.

Flavonoids: The presence of alkaloids in *V. amygdalina* was detected using Shinoda's test. Few fragments of magnesium metal were added to 3ml of extract and followed by 2 drops of concentrate HCL. Formation of magenta color indicated the presence of flavonoids.
Glycoside: Keller-Killani's test was used determine the presence of glycoside. The 5ml of extract was treated with 2ml of glacial acetic acid containing 1 drop of ferric chloride solution and 1ml of concentrated sulphuric acid. Formation of reddish brown color indicated at the junction of 2 layers which indicate the presence of a desoxy sugar characteristic of cardenolides.
Phenols: Ferric chloride's test was used determine the presence of phenols. A few drop of ferric chloride solution was added into the extracts. The presence of phenols is determined by the formation of bluish black color.
Saponin: To determine the presence of saponin, frothing test was used. 20 ml of distilled water was added to a portion of crude extracts, shaken and heating to boil. The foamy lather formation indicates the presence of saponin.
Steroids: To determine the presence of steroids, Liebermann-Burchard test was used. 2 ml extract was mixed with chloroform and 1-2 ml acetic anhydride. 2 drops of concentrated H_2SO_4 was added along the side of test tube. The blue-green ring indicates the presence of steroids.
Tannin: Lead acetate's test was used for the determination of tannin. Three drops of lead acetate solution were added to the 5 ml extracts. The formation of colored precipitate shows the presence of tannins.

Result and discussion

Table 1 summarized the morphology of bacteria strains isolated. The bacteria isolated appear in creamy white color and in straight rod-shaped.

Table 1. Characteristic of bacteria

Strain	Colony Appearance	Morphology	Gram Staining
Bacteria	Creamy white	Straight rod-shaped with rounded end	Gram-negative bacteria

Four different *V. amygdalina* leaves extracts with different concentrations was used to evaluate the *in vitro* antibacterial activity against the pathogen of pineapple heart rot disease. Table 2 below shows the *in vitro* antibacterial activity of *V. amygdalina* hexane, chloroform, methanol and aqueous leaves extracts against the pathogen of pineapple heart rot disease. Different extracts show different inhibition zone and it was agreed with previous study which stated antibacterial activity of the extracts depends on the type of solvent used (Antara & Amla, 2012).

Table 2. *In vitro* antibacterial activity of *V. Amygdalina* leaves extracts against pathogen of pineapple heart rot disease

Concentration (ppm)	Inhibition zone diameter (mm) ± Standard deviation			
	Hexane extract	Chloroform extract	Methanol extract	Aqueous extract
1000	0.0 ± 0.0	14.4 ± 2.7	3.3 ±2.0	4.4 ±1.4
5000	0.0 ± 0.0	17.5 ± 0.	5.8 ±1.3	12.8 ±0.5
10000	0.0 ± 0.0	20.7 ± 1.0	1.6 ±1.5	21.0 ±4.8
Positive control	18.5 ± 0.3	18.5 ± 0.3	18.5 ± 0.3	18.5 ± 0.3
Negative control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

In vitro antibacterial activity of *V. amygdalina* hexane leaves extracts against the pathogen of pineapple heart rot disease shows that it is not suitable to be used as a biopesticide. The test shows no inhibition zone at each concentration. *V. amygdalina* hexane leaves extracts at 1000, 5000 and 10000 ppm concentration cannot inhibit the growth of pineapple heart rot disease' pathogen. This may due to the astringent properties possessed by *V. amygdalina* hexane leaves extracts as mention by Chukwuka et al. (Chukwuka et al., 2011). Only positive control recorded the inhibition zone (18.5mm). The result may due to the absence of bioactive compounds such as flavanoid and phenol which contain antimicrobial activity and can affect the bacterial activities (Edeoga et al., 2005; Nascimento et al., 2000).

V. amygdalina chloroform leaves extracts with 10000 ppm concentration has good potential as biopesticides as it recorded the largest inhibition zone (20.7 mm) in *In vitro* antibacterial activity of *V. amygdalina* chloroform leaves extracts against the pathogen of pineapple heart rot disease. The inhibition zone recorded are more than the inhibition zone of positive control. The presence of tannin in *V. amygdalina* chloroform leaves extracts contributed to this result. Tannin has the ability to inhibit the nutrients uptake process into the microorganism' cell thus retard the growth of the microorganism (Anil et al., 2011). Other concentration (1000 ppm and 5000 ppm) also recorded good antibacterial activity as the inhibition zone more than 50% compared to positive control. *V. amygdalina* chloroform leaves extracts contain bioactive components such as alkaloid, saponin, tannin and flavanoid which has been proven to show good antibacterial activity (Odugbemi, 2006).

In vitro antibacterial activity of *V. amygdalina* methanol leaves extracts against the pathogen of pineapple heart rot disease shows the extracts has low antibacterial activity. The inhibition zone recorded is 3.3 mm (1000 ppm), 5.8 mm (5000 ppm) and 1.6 mm (10000 ppm). The varying of antibacterial results may be due to the intrinsic tolerance of the bacteria and the combination of the phytocompound in the extracts (Suree & Pana, 2005). The low antibacterial activity may also cause by the presence of least concentration of the compound that responsible for the antibacterial activity (Ahameethunisa & Hopper, 2010).

V. amygdalina aqueous leaves extracts with different concentration gives different inhibition zone. The *V. amygdalina* aqueous leaves extract showed active response towards the antibacterial activity against the pathogen of pineapple heart rot disease. The inhibition zone at 10000 ppm is larger than positive control. *Vernonia amygdalina* aqueous leaves extract contain alkaloids, tannin, and saponins which play important role in retard the bacterial activity (Rios & Recio, 2005).

Phytochemical screening test revealed that *V. amygdalina* leaves extracts shows the presence of bioactive compounds such as alkaloids, flavonoids, glycosides, phenol, saponin, steroids and tannin. Zakaria et al. (2016) also have proven that *V. amygdalina* contained flavonoids, tannin, saponin and terpenoids. Alkaloids were found in *V. amygdalina* chloroform, methanol and aqueous leaves extracts but none in *V. amygdalina* hexane leaves extracts. Most alkaloids have strong bitter taste and very toxic (Freeman & Beattie, 2008). Plants use alkaloids to defend themselves various pathogen. Phytochemical compounds found in *V. amygdalina* hexane and methanol leaves extracts in this study contrary compared to phytochemical found in a study by Oshim et al. (2016). The contrary indicated that the compounds in individual plants vary due to the climate (temperature, light, water) and growing area (Weli et al., 2014).

Table 3. Phytochemical screening test on *Vernonia amygdalina* leaves extract

	Hexane extract	Chloroform extract	Methanol extract	Aqueous extract
Alkaloids	-	+	+	+
Flavonoids	+	+	-	-
Glycosides	+	+	-	-
Phenols	-	-	+	+
Saponin	-	+	-	+
Steroid	+	-	-	-
Tannin	-	+	-	+

- absence, + presence

V. amygdalina chloroform leaves extracts shows good antibacterial activity against the pathogen of heart rot disease as it contains bioactive compounds that help in retard the microbial growth. Alkaloid and flavanoids are the bioactive substances that have antimicrobial effects (Subrahmanyam et al., 2001). While, saponin have detergent properties and plays important role in disrupting the pathogen cell membranes. Thus, the active phenolic compounds found in *V. amygdalina* chloroform leaves extracts lead to the inhibition of pathogen of pineapple heart rot disease.

The different responses may due to the age of the plants used, secondary metabolites of plants, the thickness of media agar and incorrect preparation and dosage (Calixto, 2000; Sales et al., 2016). Besides, the bacterial structures such as differences in cell wall, the amount of peptidoglycan and nature of cross-linking influence the bacterial activity (Akinjogunla et al., 2011).

Conclusion

From the result, it can be concluded that *V. Amygdalina* can be promoted as biopesticides to replace the application of synthetic pesticides. The adverse effect on the application of synthetic pesticides should be taken into serious action. Researcher and industrial people should work together to find more natural product that can be commercialize as biopesticide.

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Conflict of interests

Author hereby declares that there is no conflict of interests with any organization or financial body for supporting this research.

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